Occurrence, Growth and Survival

In this guide

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The occurrence in foods and the physiological responses in food environments for *C. botulinum*, and other clostridia that form botulinum neurotoxin, are summarised below with emphasis on the organisms that have been associated with foodborne botulism.

5.1 Spore loads in foods

Spores of *C. botulinum* and other neurotoxigenic clostridia are highly resistant to physical and chemical stresses such as desiccation, cold temperatures and weak acids and so are widely distributed in the environment. These spores can be found in the intestinal contents of animals, including humans, in soils and in both salt and freshwater sediments. It is considered impossible to be certain that unprocessed food materials do not contain spores of *C. botulinum* or other neurotoxigenic clostridia.

Dedicated surveys, using specialist detection protocols, can estimate the prevalence and the concentration of *C. botulinum* spores in food materials. In ideal conditions the best laboratory techniques, which include a non-specific enrichment step, can achieve a detection limit in the order of a few spores of *C. botulinum* per kg of food. Estimates of spore numbers are always uncertain but

surveillance also highlights significant variability associated with *C. botulinum* spore loads in different batches of food materials. Surveys of materials used in the manufacture of chilled foods, in France[156] and in the UK[157], indicate typical spore concentrations for *C. botulinum* centred around 5-10 spores per kg. Recent examination of 74 vegetable products from Finland and Germany[158] highlight load variability and estimated much higher concentrations, up to 1200 cells per kg, for one product.

Information gathered from investigation of foodborne botulism outbreaks indicates some associations between spore types and specific food sources (e.g. spores from non-proteolytic *C. botulinum* type E have been associated with fish from arctic and North American saltwater fisheries and proteolytic *C. botulinum* type A botulism is often associated with failure to correctly apply the botulinum cook during production of canned and bottled foods) but currently the relationships cannot be generalised.

A survey in the UK[159] estimated the prevalence of *C. butyricum* in a wide range of foods as 31% but did not find any neurotoxigenic strains among the isolates. Currently there is no published evidence concerning prevalence of neurotoxigenic *C. sporogenes* or *C. baratii* in food materials. Recent surveillance does not identify significant trends in the occurrence of *C. botulinum* or other neurotoxigenic clostridia but does increase confidence that, for traditional food materials, large spore loads have only small probability. It is not possible to extrapolate information from targeted surveillance studies to account for untested food materials such as alternative protein sources or specialist dehydrated dairy ingredients that might involve a concentration step in their preparation. Current surveillance methodology cannot rule out undetected loads of *C. botulinum*, with a few spores per kg, in food. The costs associated with high quality anaerobic microbiology and the significant variability observed for spore loads ensures that routine surveillance for *C. botulinum*, and other neurotoxin-forming clostridia, in food materials is impractical and uninformative.

5.2 Germination, growth and toxin production

In considering the response of *C. botulinum* and other neurotoxigenic clostridia to environmental factors, it is important to recognise that the ability to form spores and toxin introduces additional considerations for control in comparison to vegetative, non-toxigenic pathogens. To produce toxin and cause illness, the organisms must be in a vegetative state and metabolising. In many foods, particularly those subject to a heat process, the organism may only be present in

a sporulated state and must first germinate before it can produce toxin. Hence, there are potentially three ‘states’ for the organism where control can be applied; germination, growth and toxigenesis.

Historically, it was widely accepted that population growth, represented by an increase in the number of cells of the organism, occurred before toxin was produced. This was the assumption used in the 1992 report where it was stated that “growth studies can indeed be used to indicate the risk of toxin formation”. This came from research, particularly experiments performed in microbiological broth, demonstrating that toxin production occurs in late exponential phase as the organism enters stationary phase and sporulates[160]. However, published research since 1992 has demonstrated toxin production in food in the absence of measurable increases in the population size[161] so that growth may not be a reliable indicator of toxin formation. Rapid die-off of cells after toxin production could also be misinterpreted as an absence of population growth. In 2020, an ACMSF subgroup on non-proteolytic *Clostridium botulinum* and vacuum and modified atmosphere packaged foods[162] recommended that “detection of toxin is a minimum requirement for challenge testing, and that measuring viable counts is of merit in ensuring safety”. The merit in measuring viable counts is based on evidence that growth may precede toxin production and therefore when using growth and toxin production to assess the effect of controlling factors, the presence of toxin or the observation of population growth (commonly when the logarithm of the population size increases by 0.5 or more) would demonstrate unsafe conditions. The following sections on controlling factors include studies conducted on growth and/or toxin production and should be considered in light of the limitations described above.

5.3 Minimum growth temperatures

The temperature during storage affects the germination of spores and the growth of vegetative cells of *C. botulinum* and other clostridia. There are also interactions between temperature, pH, and water activity that affect growth and survival of *C. botulinum* and other clostridia. An understanding of these factors and their interaction is essential for food safety with regard to these organisms (interactions between controlling factors are discussed later in this chapter).

5.3.1 Proteolytic *C. botulinum*

Spores of proteolytic *C. botulinum* survive for long periods under normal frozen conditions, typically at –18°C, applied to foods. Proteolytic *C. botulinum* grows

between 12°C and 48°C. Optimal growth temperatures (for fastest growth) are between 35°C and 45°C. Growth at or below 10°C has been shown not to occur under otherwise optimal growth conditions and growth at 15°C is slow[163].

5.3.2 Non-proteolytic *C. botulinum*

Spores of non-proteolytic *C. botulinum* survive for long periods under normal frozen conditions, typically at -18°C, applied to foods. The temperature range for growth and toxin formation by non-proteolytic *C. botulinum*[164,165] is 3°C to 37°C, with indications for growth at higher temperature[166], but optimal growth temperatures (for fastest growth) are between 28°C and 30°C. Growth at 8°C under otherwise optimal conditions is slow.

5.3.3 Other clostridia capable of producing botulinum neurotoxin

Spores of other clostridia survive for long periods under normal frozen conditions, typically at –18°C, applied to foods. There is no evidence to distinguish the growth parameters of neurotoxigenic *C. sporogenes* from those for proteolytic *C. botulinum*. The minimum temperature for growth of non-neurotoxigenic *C. butyricum* has been reported[167] as 10°C. The minimum temperature for growth of two neurotoxigenic strains of *C. butyricum* (ATCC 43181 and 43755) in anaerobic broth are reported as 10°C and 11°C respectively. Some of six neurotoxigenic strains of *C. butyricum* involved in outbreaks of botulism in Italy, when incubated in anaerobic broth at pH = 7.0, grew and produced toxin at 12°C but not at 10°C when observed over 180 days[54]. There is only one published report on the minimum growth temperature of neurotoxigenic *C. baratii,* using a single isolate from a case of infant botulism[168,169], and this determined that growth occurred at 15°C but not at 10°C.

5.4 Thermal resistance of spores

A heating step during processing can kill spores or vegetative cells of *C. botulinum,* and other clostridia, depending on intensity (temperature) and duration.

5.4.1 Proteolytic *C. botulinum*

A meta-analysis of thermal inactivation for spores of proteolytic *C. botulinum*, including data up to 2006, gave mean value Log(D120C) = -0.78 and a standard

deviation of 0.23 for the dataset. Inverting the mean value of the logarithm gives D120C ~ 0.17 minutes but this cannot easily be identified with the arithmetic mean D-value. The analysis of D-values gave z = 10.2 centigrade degrees[170]. Another meta-analysis of thermal inactivation for spores of proteolytic *C. botulinum*[171], including data up to 2014, gave a mean value D121.1C = 0.19 minutes and standard deviation 0.11 minutes. The analysis of D-values gave z = 11.3 with standard deviation 0.3 centigrade degrees. This data was obtained in liquid medium at neutral pH and the authors considered the results were valid for temperatures 100°C and above. The meta-analyses broadly encompass the reference D-value, D121.1C = 0.21 minutes, that is generally accepted as appropriate for evaluating thermal reduction in numbers of proteolytic *C. botulinum* spores. It is also generally accepted that equivalent D-values at different temperatures are calculated using z = 10 centigrade degrees, which is similar to the z-value in one meta-analysis but at the lower end of z-values identified in the other.

A heat process resulting in 12 orders of magnitude reduction in numbers for proteolytic *C. botulinum* spores is generally considered sufficient for sterilisation of food stored at ambient temperatures over long periods. However, it should be noted that analysis of this criterion, in several studies, has moved majority opinion to conclude that a 10^{-8} - 10^{-9} probability of growth can be compared with the 12 orders of magnitude reduction in spore numbers for proteolytic *C. botulinum* in phosphate buffer that is described in the original study by Esty and Meyer[172] and is an acceptable food safety objective[162,173]. Clearly, comparison of deterministic lethality with a residual probability for growth includes a wide range of assumptions. Nevertheless the evidence does not suggest change from an established heat process of 3 minutes at 121.1°C (F0=3) to achieve a sterilisation goal for corresponding foods.

5.4.2 Non-proteolytic *C. botulinum*

In 1992 the ACMSF based their recommendations for a heat process that inactivates spores of non-proteolytic *C. botulinum* on a study which found a reference value D90C = 1.1 minutes with z = 9 centigrade degrees[174]. A meta-analysis of thermal inactivation for spores of non-proteolytic *C. botulinum*, including data up to 2006, gave a mean value Log(D120C) = -1.47 with a standard deviation of 0.71 for the dataset. Inverting the mean value of the logarithm gives D120C ~ 0.034 minutes but this cannot easily be identified with the arithmetic mean D-value. The analysis gave z = 18.6 centigrade degrees[170] but this study uses a reference temperature that is far removed from

temperatures that are traditionally used during the processing of chilled foods so that the *z*-value is difficult to interpret in terms of the safety of minimally processed foods. Another analysis of published D-values, for spores of non-proteolytic *C. botulinum* recovered in the absence of lysozyme[10], found a mean value Log(D90C) = -0.24 and standard deviation for the logarithm σ(Log(D90C)) = 0.42. The analysis gives mean value z = 6.7 with a credible range 4.4 - 10 centigrade degrees.

In 2020 the ACMSF[162] concluded that it had found evidence to recommend an update in the reference *z*-value that is included in FSA guidance documents. The recommendation indicated that z = 6.7 - 7.7 (more specifically z = 7.0) centigrade degrees should be used for calculating equivalent D-values at temperatures below 90°C whilst maintaining the reference value of z = 10 centigrade degrees for calculating equivalent D-values at temperatures above 90°C. The evidence above supports the 2020 ACMSF assessment that z = 7 centigrade degrees and z = 10 centigrade degrees should be used to evaluate equivalent heating below and above the reference temperature, 90°C, respectively and that these values should be used for evaluating equivalent thermal processes for heating temperatures in the range 83°C - 100°C.

Having considered current thermal inactivation data, whilst recognising that D-values derived from inactivation studies in food tend to be higher than those derived in laboratory medium and also the history of safety for chilled food heated at 90°C for 10 minutes or equivalent, it is clear that in the absence of other controlling factors the “90 for 10” process delivers at least a 6 orders of magnitude reduction in spore numbers, is robust and establishes confidence for control of non-proteolytic *C. botulinum* in chilled foods. However, this widely adopted reference process corresponds with a value D90C = 1.6 minutes which is longer than many measured values.

The ACMSF subgroup on non-proteolytic *Clostridium botulinum* and vacuum and modified atmosphere packaged foods[162] reviewed the evidence on the effect of lysozyme in reducing the efficacy of thermal destruction and their recommendation “that … the maximum shelf-life of foods given a heat process of 90°C for ten minutes (or equivalent) should be limited to 42 days, unless it can be shown that lysozyme is absent from the food … and that expert advice should be sought if a shelf-life in excess of 42 days is desired” remains appropriate. Challenge tests are a practical means for shelf-life extension of foods that contain lysozyme or other lytic enzymes.

5.4.3 Other clostridia capable of producing botulinum neurotoxin

There is no evidence to distinguish the thermal resistance of neurotoxigenic *C. sporogenes* from that associated with proteolytic *C. botulinum*. Neurotoxigenic *C. butyricum* (2 strains isolated from an infant botulism case) is reported to have D76.7C = 2.3 - 2.5 minutes[175] in phosphate buffer at pH = 7.0. A non-neurotoxigenic strain had a D-value 1000 times higher under the same conditions. The *z*-values for the two strains in phosphate buffer at pH = 7.0 were z = 8.2 and z = 9.5 centigrade degrees[176].

There is no published evidence detailing the heat resistance of neurotoxigenic *C. baratii*.

5.5 Effect of pH and acidity on growth and survival

Acids have an inhibitory effect on the germination and growth from spores of *C. botulinum* and other clostridia. Their inhibitory effect depends on the pH of the final food, on the concentration of the acid as well as on physical properties such as dissociation, molecular weight and the number of carboxyl groups[177]. The type of acid can influence the inhibitory effect and organic acids are more effective than mineral acids at controlling growth of microbial pathogens including *C. botulinum.* The nature of the growth medium also significantly affects growth from spores and toxin formation[178,179]. There are interactions between pH, aw and temperature that can be exploited to prevent the growth and toxin formation of *C. botulinum* and other clostridia (interactions between controlling factors are discussed later in this chapter).

5.5.1 Proteolytic *C. botulinum*

The minimum pH for growth of proteolytic *C. botulinum*[177] lies in the range pH = 4.6 - 4.8 and it is generally accepted that population growth and toxin production in foods are prohibited at or below pH = 4.6 under otherwise optimal conditions [163]. This has long been recognised as the point of demarcation for acid or acidified foods, below which *C. botulinum* is unable to grow[180]. Growth observed below pH = 4.6 has involved carefully controlled high protein conditions that are not representative of the food environment. Citric acid and hydrochloric acid tend to be less inhibitory to proteolytic *C. botulinum* than lactic acid which, in turn, is

less inhibitory than acetic acid[179].

5.5.2 Non-proteolytic *C. botulinum*

The inhibitory pH for non-proteolytic *C. botulinum* is recognised as pH = 5.0 and this is regarded as a well-established control factor[163].

5.5.3 Other clostridia capable of producing botulinum neurotoxin

Neurotoxigenic *C. butyricum* (2 strains isolated from an infant botulism case) grew and produced botulinum neurotoxin at pH = 5.2 but not at pH = 5.0, in broth acidified with citric acid[175], following incubation at 30°C. This is significantly less acid resistant than a non-neurotoxigenic strain isolated from blueberries that grew at pH = 4.2 but not at pH = 4.0 in acidified broth following incubation at 30°C. A report on growth of non-neurotoxigenic *C. butyricum* and *C. baratii* showed a pH limit in canned pasteurised mung bean sprouts of pH = 3.7 and pH = 4.0 respectively[167]. The lowest limiting pH for growth of two neurotoxigenic strains of *C. butyricum* (ATCC 43181 and 43755) in anaerobic broth was reported as pH = 4.2 using HCl as the acidulant. With other acids the limiting pH was higher; for example pH = 4.7 was limiting using citric acid and pH = 4.8 was limiting with lactic acid[176]. The results relating to organic acids indicate that growth of *C. butyricum* is inhibited in environments that are less acidic than those required for inhibition of proteolytic *C. botulinum*. In these experiments the presence of toxin was not determined and therefore it is not possible to conclude whether toxigenesis occurred. Another study showed that some of six neurotoxigenic strains of *C. butyricum* involved in outbreaks of botulism in Italy when incubated at 30°C, grew and produced toxin at pH = 4.8 but not at pH = 4.6 when the acidulant was HCl[54]. Based on current evidence, the safety controls applied to foods in relation to pH and acidity for proteolytic *C. botulinum* appear to be adequate to control the risk from neurotoxigenic *C. butyricum*, although the current data set is based on results from only a few isolated strains, and a single study indicates that neurotoxigenic *C. butyricum* shows stronger acid resistance in the presence of mineral acids.

There is no published evidence on the acid resistance of neurotoxigenic *C. baratii*.

5.6 Effect of water activity on growth and survival

The water activity of foods is affected by several different solutes; sometimes called humectants. Common humectants are sugars, glycerol and salts, particularly sodium chloride, but combinations of humectants are used widely. The lower the water activity the slower the growth of microorganisms but water activity can also have an effect on heat resistance. There are interactions between aw, pH and temperature that can be exploited to prevent the growth and neurotoxin formation by *C. botulinum* and other clostridia (interactions between controlling factors are discussed later in this chapter).

5.6.1 Proteolytic *C. botulinum*

A table of minimum water activity values allowing growth[177] indicates, for proteolytic *C. botulinum* producing type A and B neurotoxin, aw = 0.94 resulting from use of 9.4% salt and aw = 0.93 with glycerol as solute. According to ICMSF [163] most strains of proteolytic *C. botulinum* are unable to grow under otherwise optimum conditions if the salt concentration is 10% or above (aw = 0.94) and the lowest water activity allowing growth and toxin formation was observed in turkey frankfurters where use of salt resulted in aw = 0.95 and time to toxin formation was 30 days at 27°C with pH = 6.10 - 6.39.

5.6.2 Non-proteolytic *C. botulinum*

A table of minimum water activity values allowing growth indicates under otherwise optimum conditions[177], for non-proteolytic *C. botulinum* neurotoxin type E, aw = 0.97 resulting from use of 5% salt and aw = 0.94 with glycerol as the solute. Most strains of non-proteolytic *C. botulinum* are unable to grow in otherwise optimum conditions if the salt concentration is 5% or above (aw = 0.97) [163]. As temperature is reduced the minimum water activity for growth also reduces and in 1992 the ACMSF[1] stated that growth had not been reported for non-proteolytic *C. botulinum* above 3.5% salt at incubation temperatures below 10°C. However, recent tests indicate growth and neurotoxin formation in anaerobic microbiological broth with 4.5% salt at 8°C after 6 weeks, but not at 5.0% salt (aw = 0.97) at 8°C in 13 weeks[181]. It has been recognised in FSA guidance since 1992 that the safety of chilled foods can be achieved with more than 3.5% salt or water activity aw = 0.97 or less throughout all components of complex foods, and this guidance has not been associated with outbreaks of foodborne botulism in chilled foods.

5.7 Other clostridia capable of producing botulinum neurotoxin

The lowest limiting water activity for growth of two neurotoxigenic strains of *C. butyricum* (ATCC 43181 and 43755) in anaerobic broth is reported as aw = 0.96 using salt or sucrose as the humectant[176].

There is no published evidence on the minimum water activity permitting growth of neurotoxigenic *C. baratii.*

5.8 Effect of nitrite/nitrate on growth and survival of *C. botulinum*

Nitrite has an antimicrobial effect on *C. botulinum,* including the inhibition of outgrowth from spores, that is dependent on concentration, pH and a range of other factors[164]. Nitrite is commonly used for control of *C. botulinum* in chilled meats such as bacon and in ambient meats such as canned cured ham and is equally effective in inhibiting non-proteolytic[182] and proteolytic *C. botulinum*[183]*.* Acidic conditions are needed for this effect. The mechanism of action exerted by nitrite is not well understood but a review of evidence suggests that the mechanism is oxidative via the nitrite-peroxynitrite system which is pH-dependent and non-oxygen-dependent[184]. Peroxynitrite is known to be a strong oxidant and nitrating agent that causes direct oxidation or radical-mediated reactions in proteins, DNA and lipids within microbial cells. The chemical pathways involved are complex and modulated by other intrinsic and extrinsic properties of meat which, as well as the pH and redox potential, include salt concentration, heat treatment, storage temperature and addition of ascorbic acid or other reducing agents. The presence of iron in meat can also reduce the effectiveness of nitrite and this is consistent with the proposed bactericidal pathway.

The ingoing amount of nitrite is considered to contribute to the inhibitory effect rather than the residual level of nitrite. For most meat products 50 –150 mg of added nitrite (as sodium nitrite) per kg of meat is deemed to be sufficient to inhibit *C. botulinum*[164].

Nitrate does not appear to provide any anti-botulinal effect on its own but appears to act as a reservoir for nitrite formation primarily through microbiological activity in some traditionally fermented meat products[164]

although some conversion can happen through enzymatic reaction in heated cured meat products[185].

Nitrite also has bactericidal effects on other foodborne pathogens[184] so its reduction/removal should only be considered in conjunction with validation of safe shelf life. There are maximum legal limits for nitrite and nitrate in foods which differ around the world and must be adhered to because of the carcinogenic effect of nitrosamines that result from the in-process transformation of nitrites and nitrates.

There are no data on the effect of nitrite on other botulinum neurotoxin-producing clostridia and this may need further investigation to ensure their effective control.

5.9 Effect of gaseous environment and redox potential

The germination, growth and toxin production of *C. botulinum* and other clostridia in foods is affected by the gaseous environment and anaerobic conditions have traditionally been considered a pre-requisite for growth. This is because many clostridia are sensitive to oxygen and associated oxidative stress. Clostridia possess stress response genes and consequently can survive and even grow under low oxygen conditions although this does vary markedly between species, strains and depends on the medium or food in which the organism is present. The presence of high levels of oxygen does not in isolation prevent clostridia from growing or producing toxin as the cells may be protected from the effects of oxidative stress within a local microenvironment. For example *C. botulinum* can grow and produce toxin in foods packaged in air or in air permeable film[186] because the food can protect the organism from the effect of oxygen by preventing oxidation and therefore removing oxidative stress. Microenvironments are notoriously difficult to access for measurements.

As the headspace oxygen content is not a reliable indicator of potential for growth of clostridia, the oxidative state of food, measured by redox potential, has historically been used to indicate the potential to support the growth of *C. botulinum*[187,188].

Both gaseous environments and redox potential of foods can drift during the shelf life of the food due to gaseous exchange through the packaging, growth of microorganisms affecting headspace gas and redox potential or reduction/oxidation within the food matrix due to chemical reaction or

breakdown. Consequently, whilst it is reasonable to anticipate that foods packaged under vacuum or reduced oxygen may increase the potential for the growth of, and toxin production by, *C. botulinum* and other toxin-producing clostridia, it is not possible to use the gaseous atmosphere or redox potential as a controlling factor due to the inability to accurately define or control conditions necessary to prevent growth or toxin production. Lower redox potential microenvironments are difficult to access for measurement.

5.9.1 Proteolytic *C. botulinum*

Growth and toxin production by proteolytic *C. botulinum* has been reported in crumpets packaged in air[189], in laboratory media with 15% headspace oxygen[190] and in mushrooms packed in a semipermeable plastic film[191]. Toxin production occurred in overwrapped mushrooms stored under ambient conditions and it is likely that continued, post-harvest, respiration of the mushrooms during storage decreased the oxygen content making conditions more favourable for growth of, and toxin production by, *C. botulinum*.

5.9.2 Non-proteolytic *C. botulinum*

Non-proteolytic *C. botulinum* neurotoxin type E produced toxin in packaged catfish stored in oxygen permeable packaging and in an oxygen-barrier bag[192]. Similar findings were observed in Atlantic salmon[193].

5.9.3 Other clostridia capable of producing botulinum neurotoxin

Limited data are available on the effect of gaseous environment on other neurotoxigenic clostridia. Continued growth and toxin production was demonstrated[194] in anaerobic microbiological broth by *C. butyricum* type E initially grown under anaerobiosis, and then transferred to air (aerobic conditions), although this was dependent on sufficiently high number of cells, i.e. 10^3 cells per ml, being present initially.

No published evidence is available to determine the impact of gaseous conditions on neurotoxigenic *C. baratii*.

5.10 Other factors

Other factors have potential to control outgrowth and toxin production by *C. botulinum* and other neurotoxigenic clostridia. However, the efficacy is particularly variable. Some of these factors are considered in this section but others including phosphates, citrates, sorbates, sulphites, etc., which are often used in combination with other controls and cannot be evaluated for general use, are not considered further.

5.10.1 Herbs and spices

Herb and spice extracts can reduce, or in some cases inhibit, the germination, growth and toxin production by *C. botulinum* and other neurotoxigenic clostridia but the effect is highly dependent on the herb or spice, the concentration, the organism and the food matrix[195]. Oils of clove, thyme, black pepper, pimenta, garlic, onion, origanum and cinnamon have shown variable amounts of inhibitory effects on the growth of *C. botulinum* producing type A, B and E neurotoxins in anaerobic microbiological broth[196]. Alcoholic extracts from 33 herbs and spices have a range of minimum inhibitory concentrations for *C. botulinum* (neurotoxin types not reported) in anaerobic microbiological broth[197]; these included mace, nutmeg, bayleaf, black and white pepper, paprika, rosemary, cloves, oregano, turmeric and thyme. Some extracts showed little or no inhibition even at high concentration; these included parsley, mustard, garlic, celery, chives, fennel, tarragon, dill, cumin, onion and coriander seed. The inhibitory effect of spices such as oregano, savory and thyme are potentially due to the high content of carvacrol and thymol but this can vary in concentration between different varieties and regions and is dependent on growing conditions[198].

There is no published evidence detailing the effect of herbs and spices on growth and toxin production for neurotoxigenic *C. butyricum* or *C. baratii.*

Whilst it is possible that specific herb and spice extracts may be able to inhibit *C. botulinum* in vitro little is known about the effect in food matrices[199]. Extracts that migrate to the oil phase of complex foods will no longer be active in the water phase. Without specific reproducible evidence it is not possible to consider the generic use of herb or spice extracts as a control factor for *C. botulinum* and other neurotoxin-producing clostridia.

5.10.2 Nisin

Nisin is a natural antimicrobial peptide produced by the lactic acid bacterium *Lactococcus lactis* ssp. *lactis*. It has a spectrum of activity against a number of Gram-positive bacteria including many clostridia. Nisin is authorised for use in a

number of products throughout the world and, in the EU and the UK, levels are defined in legislation[200,201]. In the EU (and UK) nisin is approved for use in a number of dairy products including cream, cheese, some dairy desserts and also in egg products at inclusion levels ranging from 3mg/litre (or kg) to 12.5 mg/litre (or kg). Nisin is most effective at acidic pH values in food and it shows less efficacy in meats due to inactivation by glutathione and proteolytic enzymes together with interactions with phospholipids[202]. High levels are required to prevent growth and toxin production by *C. botulinum* (200-400 mg/kg) but, commercially it is generally used with other controlling factors at much lower levels i.e. 12.5mg/kg in processed cheeses. In this way nisin can replace other factors traditionally used in the control of *C. botulinum* such as sodium chloride.

Processed cheese inoculated with proteolytic *C. botulinum* types A and B and stored at 30°C for 48 weeks did not support toxin production when 12.5mg/kg nisin was used to replace 2.0% sodium chloride[203].

Non-proteolytic *C. botulinum* has been shown to be more sensitive to nisin than proteolytic *C. botulinum*[204].

There is no published evidence on the effect of nisin on neurotoxigenic *C. butyricum* and *C. baratii*.

5.10.3 Competitive microflora

The natural microflora of a food product can influence the growth and toxin production of *C. botulinum*. This may result from enhancing the growth potential by creating microenvironments in foods where growth, that would ordinarily be inhibited, could occur. Mould growth in tomato juice was shown to create conditions suitable for growth and toxin production by proteolytic *C. botulinum* types A and B although spoilage was evident[205,206] (similar conditions could arise from growth of *Bacillus* spp.). Microflora can inhibit growth and toxin production by *C. botulinum* due to competitive effects or through the production of inhibitory compounds including acids and other antimicrobials. This effect has been reported for proteolytic *C. botulinum* types A and B in experimental meals [207]. For non-proteolytic *C. botulinum* type E the specific inhibitory effects of the microflora were not clear and it was established that spoilage due to the microflora occurred prior to, or at the same time as, neurotoxin production[208,209]. Lactic acid bacteria have specific anti-botulinum properties against both proteolytic and non-proteolytic *C. botulinum* due to the production of bacteriocins [210] and this had led to the development of compounds suitable for use as specific inhibitors such as nisin (the properties of nisin are described earlier in this

chapter). The effect of lactic acid bacteria on the growth of non-proteolytic *C. botulinum* is reported as insignificant[211].

The use of defined cultures in the production of some fermented foods, such as yogurt and cheese, where known and measurable inhibitory compounds such as organic acids are produced are recognised methods used to achieve control of *C. botulinum* and other botulinum neurotoxin-producing clostridia.

There is no evidence detailing the effect of competitive microflora on other botulinum toxin-producing clostridia including *C. butyricum* and *C. baratii.*

Reliable control of *C. botulinum* and other neurotoxigenic clostridia using competitive microflora cannot be achieved and is not recommended outside the use of known cultures creating specific, defined and measurable inhibitory compounds in fermented products.

5.11 Combination of factors

The factors that are known to inhibit the germination and growth from spores of *C. botulinum* can be combined in many ways to ensure safety of food with respect to botulism. Combining multiple controls adds important flexibility to product development processes but also adds complexity to food safety considerations. Combinations of factors may cause growth rates, or inactivation rates, to change, or may modify the limits for which growth can occur, compared to the values established by single factor tests. Combined controls must be evaluated carefully using predictive microbial models and/or dedicated challenge tests. It is important to appreciate that the controlling factors, considered individually or in combination, relate to bacterial population growth whereas food safety is concerned with botulinum neurotoxin formation.

Predictive microbial models have become increasingly sophisticated and some developments, such as Combase[212] and the Pathogen Modelling Program[213] (PMP), have become large versatile online resources with integrated tools to make them easily accessible and user friendly. Advanced modelling includes non-linear (one step) fitting of responses, complex constructions for multidimensional growth boundaries and statistical tools to evaluate confidence in predictions etc. The fundamentals of predictive microbial modelling, including construction, validation and applications have been reviewed in the context of microbial risk assessment[214].

Predictive models for the survival and growth of *C. botulinum* are particularly challenging. Difficulties associated with *C. botulinum* microbiology mean that the corresponding predictive models often concentrate on population growth (not toxin production) and are sometimes based on experiments that use non-toxigenic strains as surrogates (often with unnaturally large inocula) so they can be criticised as unrepresentative. Where predictive models are used to consider extending shelf life beyond 10 days any indication of population growth must be interpreted as also indicating neurotoxin formation and therefore that the scenario is hazardous.

In addition, *C. botulinum* models are often built from data generated in laboratory media that do not closely resemble real foods, and do not include competitive microflora, so can be considered incomplete.

Predictive microbial models are dominantly deterministic whereas it is now accepted that botulism hazards may involve significant stochastic processes surrounding germination or inactivation of individual spores and particularly heat treated spores[215]. There is very little structured information about the effects of environmental factors in relation to the stochastic elements of spore responses so that interpretation of outputs from deterministic models requires specialist *C. botulinum* expertise.

Combase and the PMP include models, parameterised by temperature, pH and aw/salt, that predict the time to growth from spores for proteolytic and for non-proteolytic *C. botulinum*. These models have become well established, and are regarded as fail-safe in most conditions that correspond with processing and storage of real foods, but they are inflexible and therefore limited in their applications. Specialised models, such as that developed for growth from proteolytic *C. botulinum* spores during dynamic (cooling) temperature regimes in ground beef[216] or that developed to account for the effects of multiple food preservatives (organic acids) on growth from non-proteolytic *C. botulinum* in minimally processed fish or chicken[217], extend the range of applications but are less well established and therefore they are not so easy to implement and evaluate. Expert interpretation is essential for predictive models that relate to *C. botulinum* in food and their use in product development, shelf-life optimisation, timely decision making or in progression of HACCP based safety management.

Challenge tests that explore the possibility of toxin production by *C. botulinum* in real foods are an essential element of safety evaluations. *Clostridium botulinum* challenge tests are an integral part of the development cycle for large groups of foods including minimally processed chilled foods and low acid foods stored at

ambient temperatures (often in modified atmospheres). Multifactorial predictive microbial models for *C. botulinum* may effectively guide and support the efficient use of challenge tests in safety evaluations and in product developments. Protocols for challenge tests involving *C. botulinum* have recently been considered by the ACMSF[162]. The ACMSF advised that all challenge tests involving *C. botulinum* should centre on detection of neurotoxin and that, even in the absence of toxin, any observation of population growth for *C. botulinum* in food should be considered potentially hazardous. Tests centre on detection of botulinum neurotoxin as published reports indicate that population growth may not be a reliable indicator of neurotoxin formation and foodborne botulism is an intoxication caused by the neurotoxin. Evidence indicates that strong population growth (e.g. completed exponential phase) in food is associated with detection of neurotoxin formation, although initial detection of population growth may in some circumstances precede detection of neurotoxin. This may result in some challenge test studies where growth is detected in the absence of neurotoxin if the study is terminated in the intervening period. The botulinum neurotoxin appears stable in food environments relevant to challenge tests[218], but may be inactivated by heat or at high pH not found in food[182]. Detection of neurotoxin relies on effective extraction (that should be confirmed through the use of appropriate controls) followed by sensitive and specific detection (detection methods are described in Chapter 3 of this report).

Validated models for inactivation of spores, for *C. botulinum*, are almost exclusively linear and account for temperature as a single lethal factor. Additional dependency of thermal inactivation rates on other physico-chemical properties of food, such as pH or fat content, have been observed[219] but are not routinely included in probabilistic models of thermal death for *C. botulinum*.

There are no published models for growth or inactivation of neurotoxigenic strains of *C. butyricum* or *C. baratii*.

5.12 Novel processes

Traditional food safety technologies, including stringent heat processes and freezing, can negatively affect perceived product quality. Since 1992 there has been a consistent search for more complex preservative technologies that can maintain product safety with respect to botulism whilst enhancing organoleptic properties of food. These include novel thermal and non-thermal technologies.

5.12.1 Thermal technologies

Ohmic heating, in which alternating electrical current is passed through, heats up food[220] and rapidly destroys the cell membrane of bacteria leading to lethal leakage of bacterial cellular contents. This process has the advantage of being much faster than conventional steam heating[221]. Since ohmic heating works in water continuous systems, and relies on electrical conductivity, foods containing particles with low conductivity, such as oil droplets, require validation to ensure minimum temperatures are achieved throughout the food being heated.

High-frequency heating uses a radio frequency heating system to induce molecular friction and heat to inactivate pathogens[222,223]. Although applications have been developed for industrial drying and thawing frozen food, the non-uniform heating caused by uneven distribution of electromagnetic fields in foods mean that more in-depth simulations and investigations are required before it can be applied more widely[224]. Although effects on clostridial spores are not recorded, inactivation of spores from other spore-forming bacteria such as *Bacillus* spp. is reported and application of this technology in the future would likely utilise existing thermal processing data, such as D and *z*-values, for clostridial spores.

Power ultrasound/thermosonication uses pressure waves, with frequencies 20-100kHz, to cause cavitation, generation of hydroxyl radicals and bacterial death [225]. Currently, applications focus on the use of this technology for washing and decontaminating fresh foods and reducing the microbiological load. The focus of studies investigating use of this technology is on vegetative pathogens, not bacterial spores. Further research is required if this technology is to be applied as an intervention technique in the same way that thermal processes are used for inactivating clostridial spores in foods that are processed in-line or in-pack.

Microwave thermal sterilisation, a two-stage process, combines microwave heating with thermal sterilisation with water as an initial heating medium and is considered as an option to destroy clostridial spores and cells while preserving desirable food attributes[226,227]. Using microwaves to heat water that is then used to heat foods in-pack allows for more uniform heating, avoiding the problems caused by non-uniformity of electromagnetic fields and identification of ‘cold spots’. This process technology utilises existing thermal processing data, such as D and *z*-values, for spores of *C. botulinum*. However, the handling of process deviations is more complex than in conventional thermal processes[226].

5.12.2 Non-thermal technologies

High pressure processing (HPP), involving pressures between 100 and 1000MPa, is used widely, especially to inactivate vegetative spoilage organisms and vegetative foodborne pathogens, in meats, fruit juice and seafood. Pressure treatment alone does not achieve substantial reductions in bacterial spores. However, changes in pressure are implicitly and explicitly linked to heating[228] and HPP is frequently used in combination with other controls for bacterial spores such as refrigeration and low pH [229,230]. For non-proteolytic *C. botulinum* type E spores, there is a report of high pressure (900 and 1200 MPa) delivering a 6 log inactivation at 60°C and 75°C after 10 min[231]. For a single strain of proteolytic *C. botulinum* type B, however, pressure-mediated protection of spores has been reported[232] and there appears to be significant variation in susceptibility between different strains of proteolytic *C. botulinum*[231]. Pressure-assisted thermal sterilisation (PATS) has been certified by the US FDA, involving the use of a chamber between 60°C and 90°C that as a result of internal compression heating (using 600 MPa pressure, or higher), leads to in-process temperatures reaching 90°C -130°C very quickly. High pressure treatment times for the inactivation of *C. botulinum* can be shorter than for traditional heating but there is no single safe process[230].

Pulsed light uses high-frequency light pulses, typically 200–1,100 nm[233], but the efficiency of this technology has been principally confirmed in liquid foods[221]. Ultraviolet light treatment is commonly used as an antibacterial strategy, particularly for inactivation of vegetative bacteria, but is practically ineffective for inactivating spores[234].

Cold plasma technology generates reactive oxygen and nitrogen species, and UV radiation which can inactivate bacteria, fungi and viruses[235]. It is suggested as a potentially important element in multiple hurdle technologies to decontaminate foods and food packaging surfaces[224,236]. However, the antimicrobial efficacy is diminished by roughness of the product surface and prediction of the plasma reaction chemistry is made difficult by the wide range of moisture concentration in foods[237]. The mechanisms by which cold plasma inactivates bacterial spores have not yet been fully elucidated[237]. Additional uncertainties related to the parameters and application of cold plasma technology are still outstanding[238, 239,240] and application as an alternative intervention process for delivering defined log reductions of clostridial spores in foods is not currently possible.

5.12.3 Packaging technologies

Since 1992 developments include smart or active packaging materials that contain additives that can extend the quality or shelf life of foods. Packaging materials may include antimicrobials, antioxidants, light blockers[241] and oxygen scavengers, carbon dioxide emitters and moisture regulators[242]. "Intelligent packaging" can monitor and provide information on the current quality, freshness, maturity, time-temperature status and leakage/contamination of products during transport, retail and domestic storage[242–245]. Changing consumer perceptions regarding renewable and environmental-friendly packaging [246] mean there are significant moves away from single use petroleum-based plastic in food packaging to a range of biodegradable natural-based polymers[243]. Compared to oil-based plastic packaging materials bio-based films are desirably biodegradable but have low thermal stability and increased water sensitivity. Research is needed to evaluate the safety and possible unintended consequences when novel materials are used in recyclable food packaging[247,248]. These characteristics are of particular concern in relation to possible *C. botulinum* growth and neurotoxin production on/in biobased packaging materials at refrigeration temperature[249].

Some concerns and/or uncertainties remain in relation to the safety and effectiveness of packaging materials of some systems[250,251,252]. The FDA has recently developed a control strategy for reducing the hazard of *C. botulinum* on seafood packages by using time and temperature integrators[253]. EFSA has recommended rigorous tests before approving the use of some active packaging systems.

5.13 Conclusions

There is no evidence to suggest that spore loads in food materials have changed substantially since 1992 but dedicated surveillance has added confidence to the belief that high spore loads have small probability. A small number of observations emphasise the role of variability in *C. botulinum* spore loads for food materials. The occurrence of neurotoxigenic clostridia other than *C. botulinum* is poorly characterised.

Some control factors that prevent outgrowth from spores or population growth and toxin formation, by *C. botulinum* or other neurotoxin-producing clostridia, are well established and some relevant parameters are collected in Table 6. In particular heating at 90°C for 10 minutes is a robust process to deliver at least a 6 order of magnitude reduction in spore numbers, for non-proteolytic *C. botulinum.* However, this widely adopted reference process corresponds with a

value D90C = 1.6 minutes which is longer than many measured values. Equivalent lethality of thermal processes can be calculated with z = 7 centigrade degrees and z = 10 centigrade degrees below and above the reference temperature respectively; some values are indicated in Table 7. Water activity is a particularly complex control factor because the responses of *C. botulinum*, and other neurotoxigenic clostridia, depend on the solute used as a humectant as well as the value of the available water.

Neurotoxigenic *C. butyricum* appears equally or even more susceptible to recognised controls than proteolytic *C. botulinum* although stronger acid resistance in the presence of mineral acids is an exception. There is only a small amount of evidence to quantify the physiological responses of neurotoxigenic clostridia other than *C. botulinum*.

There is some information available on the impact and implications of some novel thermal and non-thermal technologies available to inactivate undesirable bacteria or spores during food processing. However, most of the sources of evidence note the need for further research to confirm consistently safe, robust, effective and commercial valid use of these technologies. For novel thermal processes thorough validation to establish that a minimum temperature is achieved throughout the food is crucial.

While the advantages in using active (and intelligent) packaging of food are clearly increasing, significantly reducing food wastage and limiting environmental impact, some concerns and/or uncertainties remain in relation to safety.

Table 6

Controlling factors for Clostridium botulinum and other botulinum neurotoxin-producing clostridia in foods and recommended safe processes[a]

Proteolytic *C. botulinum*	**Non-proteolytic *C. botulinum***	***C. butyricum* (neurotoxigenic)**	***C. baratii* (neurotoxigenic)**

Minimum growth temperature	Value	10°C – 12°C	3°C	10°C	10°C – 15°C
Minimum growth temperature	Generally Adopted Control	Store foods at less than 10°C	Store foods at less than 3°C	Store foods at less than 10°C	Store foods at less than 10°C
Thermal destruction of spores	Value	D121.1°C = 0.19-0.21 minutes	D90°C = 1.1 minutes	D76.7°C = 2.3 – 2.5 minutes	NR
Thermal destruction of spores	Generally Adopted Control	Heat process low acid ambient foods pH > 4.6 at 121.1°C for 3 minutes or equivalent using z = 10 centigrade degrees	Heat process foods intended to be chilled (8°C or less) with extended life (greater than 10 days) at 90°C for 10 minutes or equivalent[b]	Heat processes for proteolytic and non-proteolytic *C. botulinum* would deliver safety for neurotoxigenic *C. butyricum*	NGAC
Inhibitory pH	Value	4.6	5.0	4.7	NR

Inhibitory pH	Generally Adopted Control	Formulate to pH = 4.6 or less throughout the food[c]	Formulate to pH = 5.0 or less throughout the food[c]	Formulate to pH = 4.6 or less throughout the food[c]	NGAC
Inhibitory water activity (with salt)	Value	0.94	0.97 (0.97 at 8°C)	0.96	NR
Inhibitory water activity (with salt)	Generally Adopted Control	Formulate to aw = 0.94 or less throughout the food[d]	Formulate to aw = 0.97 or less (chilled) throughout the food[d]	Formulate to aw 0.96 or less throughout the food[d]	NGAC
Inhibitory water activity (with glycerol)	Value	0.93	0.94	NR	NR
Inhibitory water activity (with glycerol)	Generally Adopted Control	Formulate to aw = 0.93 or less throughout the food	Formulate to aw = 0.97 or less (chilled) throughout the food	NGAC	NGAC
Inhibitory salt concentration (aqueous)	Limit	10%	5% (4.5% at 8°C)	NR	NR

Inhibitory salt concentration (aqueous)	Generally Adopted Control	Formulate to 10% or greater aqueous salt throughout the food.	Formulate chilled food to 3.5% or greater aqueous salt throughout.	NGAC	NGAC

NR: Not reported

NGAC: No generally adopted control

[a] The figures detailed in the rows titled ‘Value’ are derived from the scientific literature (and are discussed further in the text of this report) under otherwise optimal conditions for growth and, on occasion, indicate growth and / or toxin production beyond established norms that are specified in the rows titled ‘Generally Adopted Control’.

[b] Calculate equivalent thermal processes using z = 7 for process temperatures below 90°C and z = 10 for process temperatures above 90°C

[c] Ensure that precautions are taken to prevent contamination and growth of microorganisms that could elevate pH e.g. moulds, butyric anaerobes, bacilli

[d] If using humectants other than sodium chloride or glycerol, lower values of aw may be necessary

Table 7

Isothermal processes that have equivalent lethality to the reference process, 10 minutes at 90°C, for non-proteolytic C. botulinum

Temperature (°C)	Time (min)
100	1.0

99	1.3
98	1.6
97	2.0
96	2.5
95	3.2
94	4.0
93	5.0
92	6.3
91	7.9
90	10.0
89	13.9
88	19.3
87	26.8
86	37.3
85	51.8
84	72.0

83	100.0